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INDIRECT DETECTION OF ANTIBIOTICS IN MILK

R. E. HARGROVE

EASTERN UTILIZATION RESEARCH AND DEVELOPMENT DIVISION,
UNITED STATES DEPARTMENT OF AGRICULTURE,
WASHINGTON, D.C., U.S.A.

and

R. D. PLOWMAN

ANIMAL HUSBANDRY RESEARCH DIVISION,
UNITED STATES DEPARTMENT OF AGRICULTURE,
BELTSVILLE, MD., U.S.A.

INTRODUCTION

NUMEROUS market-milk surveys have shown that residual veterinary antibiotics frequently enter the general milk supply. These antibiotics may, and occasionally do, occur in sufficient concentrations to inhibit or prevent the growth of bacterial starters used in the manufacture of cheese and fermented milks, thus causing serious economic loss.

Although several sensitive and apparently reliable tests have been proposed for the detection of antibiotics in milk, all of them are laboratory tests that require from 2½ to 8 h. The time, equipment and skills required to perform these tests reduce their usefulness to cheese-makers and milk-plant operators.

If the dairy farmer could be provided with a practical means whereby he could detect the antibiotic as it appears in the milk of a treated animal, he would be less apt to include such milk in his supply. With a rapid and reliable means of detection the manufacturer could detect antibiotic-containing milk as it is received at the plant and thereby divert such milks from his operation.

Several workers have suggested that dyes or colouring materials be added to veterinary antibiotics to colour the milk after mastitis treatment. However, very few reports have been published on the subject. Dalgaard-Mikkelsen & Rasmussen (2) in 1957 suggested the use of a green tracer dye called "Green S" to identify antibiotic-containing milks. Hargrove *et al.* (1) in 1958 reported on the use of a fluorescent marker added to veterinary antibiotics to indirectly detect antibiotic-containing milks. The present report summarizes the earlier work and includes the results of recent studies.

EXPERIMENTAL

Screening of compounds for marker properties

The compounds screened for possible use as markers included certified food colours, cosmetic dyes, food flavours, odoriferous materials and various fluorescing substances, such as the fluoresceins, chlorophylls and hydroxycoumarins. Each material was tested for ease and limit of detection in whole milk. Milks containing fluorescent materials were diluted serially and measured for fluorescence with an inexpensive 2-amp, long-wave (3660Å), ultra-violet lamp. Compounds that showed promise were tested further by udder infusion in conjunction with penicillin.

Udder infusions

Three separate trials were conducted to evaluate the marker materials by udder infusion. Each trial was designed as a balanced incomplete block experiment. Four markers were assigned at random to the 4 quarters of the udder of 6 cows representing different levels in milk production. Quarter milkers were used to collect samples of milk from individual quarters for at least 96 h after infusion. Close veterinary scrutiny and milk-production records were maintained. Milk samples were tested for leucocytes, pH, antibiotic content and presence of marker. Milks containing marker were diluted with herd milk to determine the extent of dilution without loss of marker detection. Some markers were subjected to additional trials, if further testing seemed advisable.

Correlation study

An attempt was made to correlate the persistence of penicillin and the markers in the udders of the cows. Data accumulated from the udder-infusion experiments were statistically analysed to evaluate the marker materials, as well as to determine the effect of the level of milk production on the rate of excretion of antibiotic and marker from the udder. Correlation coefficients for penicillin and marker persistency in the udders were determined for the total number of milkings and also within milkings. On the basis of statistical data, a combination of oil-soluble fluorescein (Fluoral) * and uranine (sodium fluorescein) was found to be the most suitable marker of those tested, and it was selected for further study and evaluation.

Marker-antibiotic storage study

The effect of marker on the antibiotic activity of various types of veterinary antibiotics during storage was studied. Ten different commercial preparations containing multiple antibiotics were selected for the storage tests. The antibiotics represented in the preparations were penicillin, dihydrostreptomycin, bacitracin, neomycin, polymyxin, erythromycin, chlortetracycline and streptomycin. Sulpha drugs were also in

* General Dyestuff Company.

The use of trade names is for the purpose of identification only, and does not imply endorsement of the product or its manufacturer by the U.S. Department of Agriculture.

several of these preparations. The marker was added to each preparation at the rate of 250 mg of oil-fluorescein and 125 mg of uranine per unit dose and the mixtures were stored at room temperature. Each preparation was assayed quantitatively for its antibiotic activity at monthly intervals.

Test for marker toxicity

Two methods were followed to determine if increased doses of marker might have a toxic effect on the cows. In one instance the marker was added to a veterinary preparation at the rate of 250 mg of oil-fluorescein and 125 mg of uranine per dose. Three cows were treated with this preparation for four successive milkings. In a second procedure massive doses of the marker were injected into the udders of 6 cows. Dosages ranged from 8 to 16 times the normal amount (1–2 g/dose). In each method the cows were observed for evidence of toxicity for at least 96 h after infusion.

Persistence of marker in the udder

Eight commercial veterinary antibiotic preparations were selected to be tested by udder infusion with added marker to determine if the rate of marker excretion and persistence in the udder might vary among the different types of preparations. The vehicles represented in the preparations were mineral oil, sesame oil, peanut oil and lanolin. The prescribed dosages on the labels ranged from 6 to 28 ml. Four cows were injected with the 8 preparations in 2 trials. The experiment was designed so that each cow received all 8 treatments in 2 periods with no quarter receiving the same treatment twice. Milk samples were collected for 96 h after infusion and tested for marker content. Data from these trials were analysed statistically.

Mastitis treatment

Eighteen cases of mastitis were treated with 9 different commercial antibiotics to which the fluorescein marker was added. Two cows were treated with each preparation. Milk samples were collected for 96 h after treatment, measured for marker content and assayed for each of the antibiotics that were in the original preparation. The values obtained were used to correlate the persistence of marker and antibiotic in the udder.

RESULTS

The results of these studies are summarized briefly as follows:

(i) In general the odorous and flavoured compounds were unsatisfactory as markers because of poor detectability.

(ii) The fluorescing compounds were superior to the other materials tested with respect to ease and limit of detection in whole milk. Oil-fluorescein and uranine was detectable in milk by ultra-violet light in a dilution of 1 p.p.m.

(iii) Oil-chlorophyll, uranine and oil-fluorescein were readily detected in the milk by visual inspection as it was withdrawn from a treated animal. No evidence of

toxicity to the cows was noted with oil-fluorescein, uranine, oil-chlorophyll or esculin. A combination of oil-fluorescein and uranine coloured both the fat and non-fat portions of the milk.

(iv) A combination of 125 mg of oil-fluorescein and 125 mg of uranine showed a greater correlation with the excretion of penicillin than did any other test material. It coloured the milk visually for 48 h and could be detected with ultra-violet light up to 96 h after treatment. Fig. 1 presents representative data which show a close correlation in the excretion of penicillin and the fluorescein marker. It was found that the milks containing about 10 units of penicillin per ml could be diluted 100 times and still have the marker detectable by ultra-violet light.

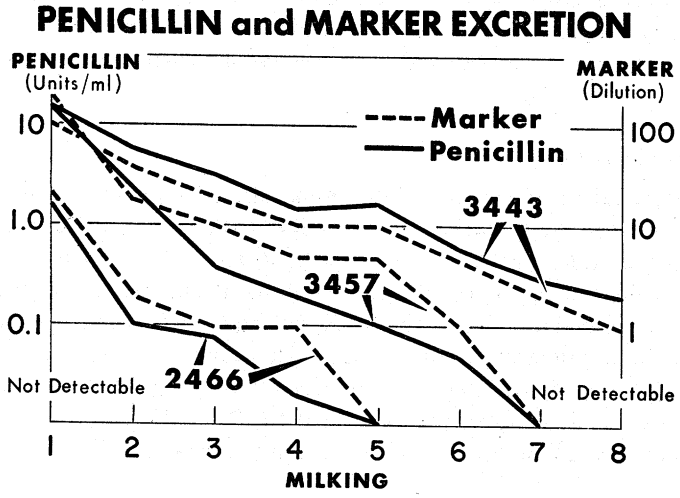

Fig. 1.

(v) No appreciable loss of antibiotic activity was detected over a 5-month storage period with 8 commercial antibiotics containing the marker. However, 2 additional preparations showed a marked decrease in antibiotic activity. The antibiotics that showed a decrease in the 2 products appeared to be stable in the other 8 preparations, which suggests something more than an antagonism between marker and antibiotic.

(vi) No evidence of a toxic reaction to the cows was noted following 4 successive treatments of the marker-penicillin preparation or by massive doses of marker up to 1 g dose (8 times the normal). Two out of 4 cows developed a hard quarter shortly after infusion with massive doses of 2 g of marker (16 times the normal dose).

(vii) No marked difference was found between the rates of markers excretion from 8 different commercial antibiotic preparations. The retention of both marker and antibiotic was influenced greatly by the level of milk production. Low-producing cows retained penicillin and marker as long as 24–48 h, and in higher concentrations, than did the high producers.

(viii) Considerable variation was found in the retention of various antibiotics, when mastitic cows were treated with multiple-type antibiotics. However, in all cases the marker could be detected as long as any antibiotic was present. Fig. 2

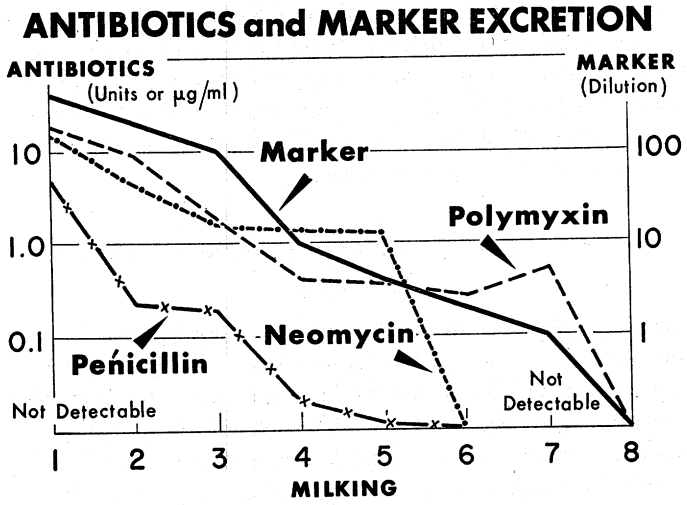

Fig. 2.

represents a mastitis case that was treated with a multiple antibiotic that contained added marker.

CONCLUSION

In view of the results obtained in this study, it appears that the addition of fluorescent tracer-dyes to veterinary antibiotics should provide rapid and practical means of indirectly detecting antibiotics in milk. A combination of 125 mg each of oil-fluorescein and uranine added to veterinary antibiotics intended for intramammary infusion was superior to the other materials tested.

REFERENCES

(1) Hargrove, R. E., Lehman, R. J. & Matthews, C. A. (1958) *J. Dairy Sci.* **41** 617
(2) Dalgaard-Mikkelsen, S. & Rasmussen, F. (1957) *Nord. VetMed.* **9** 852

SUMMARY

A number of fluorescent materials, such as fluorescein, chlorophyll and esculin, were added as a "marker" to veterinary antibiotics, infused into the udders of cows and tested for their ability to indirectly indicate the presence of antibiotics in the milk. The fluorescent marker materials were statistically evaluated on the basis of data obtained from antibiotic assays and marker determinations made on the milks from the treated animals for 96 h after infusion.

A combination of 125 mg each of oil-soluble fluorescein (Fluoral) and uranine (sodium fluorescein) showed a closer correlation with the excretion of penicillin from

the udder than did the other test materials. The marker was detected visually in the milk from the treated quarters by its yellow-green colour for 48 h, and could be detected with an inexpensive long-wave ultra-violet lamp (2 amp 3660 Å) up to 96 h after infusion. The marker coloured both the fat and non-fat portions of the milk.

The level of milk production greatly affected the excretion of both markers and antibiotics from the udder. No evidence of toxic effects to the cows or their udders was detected by the infusion of the prescribed dosages of marker. No appreciable loss of antibiotic activity was found in 8 different commercial antibiotic preparations when mixtures of marker and antibiotic were stored at room temperature. However, two preparations showed a marked loss in activity.

DETECTION INDIRECT DES ANTIBIOTIQUES DANS LE LAIT

RESUME

Certaines matières fluerescentes, telles que la fluorescéine, la chlorophylle et l'esculine, étaient ajactées en tant que «témoins» aux antibiotiqùes vétérinaires. On en a infusé au pis des vaches afin d'étudier leur aptitude à indique indirectement la présence des antibiotiques dans le lait. Une évaluation statistique des matières-témoins fluorescentes fut établie en partant des résultats obtenus en analysant pendant 96 h suivant l'infusion le contenu en antibiotiques et en matières-témoins du lait des animaux traités.

Le mélange de 125 mg de fluorescéine soluble dans l'huile (Fluoral) et de 125 mg d'uranine (fluorescéine de soude) a présenté un plus fort rapport avec l'excrétion de la pénicilline des tétines que les autres matières étudiés. Grâce à sa couleur jaune-verte, le témoin marquait clairement le lait des animaux traités et restait visible pendant 48 h; on pouvait déterminer sa présence même 96 h après l'infusion en se servant d'une lampe ultraviolette bon marché à longues ondes (2 amp, 3660 Å). Le témoin coloriait tant les parties grasses que non-grasses du lait.

Le niveau de la production du lait influençait très fortenait l'excrétion des matières-témoins et des antibiotiques des pis. On n'a découvert aucune indication d'un effet toxique que l'infusion des doses prescrites de la matière-témoin aurait pu avoir sur les vaches ou sur les pis. On n'a pas observé de perte appréciable de l'action antibiotique dans le cas de huit préparations antibiotiques commerciales différentes, lorsque le mélange du témoin et des antibiotiques était préservé au température du chambre. Cependant, on a observé une perte considérable en activité en le cas de deux préparations.

INDIREKTE BESTIMMUNG VON ANTIBIOTICA IN MILCH

ZUSAMMENFASSUNG

Man fügte eine Anzahl von fluoreszenen Stoffen, Chlorophyll und Esculin als „Merker" tierärztlichen Antibiotics bei, führte sie in die Euter von Kühen ein, und prüfte sie auf ihre Fähigkeit hin, indirekt das Vorhandensein von Antibiotics in der Milch festzustellen. Man hat die fluoreszenen Merkerstoffe statistisch auf der Grundlage gewertet, die von Data antibiotischen Probierguts und Merkerfeststellungen von

der Milch der behandelten Tiere durch 96 Stunden hindurch nach deren Einführung vorgenommen wurden.

Eine Konbination von je 125 mg fettauflösenden Fluorszein (Fluoral) und Uranin (Sodium Fluoreszein) zeigten einen engeren Zusammenhang der Ausscheidung von Penicillin durch das Euter, als die anderen verwendeten Probestoffe an. Der Merker konnte mit freien Auge in der Milch der behandelten Tiere durch seine gelbgrüne Farbe durch 48 Stunden hindurch beobachtet werden und konnte dann mit Hilfe einer billigen ultravioletten Langwellenbirne (2 amp, 3660 Å) bis zu 96 Stunden nach Einführung nachgewiesen werden. Der Merker färbte die Fett-, sowie auch die Nichtfettbestandteile der Milch.

Sehr stark beeinflusste das Niveau der Milchproduktion die Ausscheidung der beiden Merker und Antibiotics durch das Euter. Keinerlei Anzeichen von toxischen Begleiterscheinungen konnten an den Kühen oder an den Eutern nachgewiesen werden, solange die Einführungen in den vorgeschriebenen Dosen für Merker stattfand. Kein nennenswerter Verlust antibiotischer Wirksamkeit konnte in den acht verschiedenen Präperaten nachgewiesen werden, solange die Mischung von Merker und Antibiotics in Zimmertemperatur aufbewahrt wurden. Jedoch, zwei Präperate zeigten einen bedeutenden Verlust in Wirksamkeit.